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papA Gene of Avian Pathogenic Escherichia coli

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Abstract
P fimbrial adhesins may be associated with the virulence of avian pathogenic *Escherichia coli* (APEC). However, most APECs are unable to express P fimbriae even when they are grown under conditions that favor P fimbrial expression. This failure can be explained by the complete absence of the *pap* operon or the presence of an incomplete *pap* operon in Pap-negative APEC strains. In the present study, we analyzed the *pap* operon, specifically the*papA* gene that encodes the major fimbrial shaft, to better understand the *pap* gene cluster at the genetic level. First, by PCR, we examined a collection of 500 APEC strains for the presence of 11 genes comprising the *pap* operon. Except for *papA*, all the other genes of the operon were present in 38% to 41.2% of APEC, whereas the *papA* was present only in 10.4% of the APEC tested. Using multiplex PCR to probe for allelic variants of *papA*, we sought to determine if the low prevalence of *papA* among APEC was related to genetic heterogeneity of the gene itself. It was determined that the *papA* of APEC always belongs to the F11 allelic variant. Finally, we sequenced the '*papA* region' from two *papA*-negative strains, both of which contain all the other genes of the *pap* operon. Interestingly, both strains had an 11,104-bp contig interrupting *papA* at the 281-bp position. This contig harbored a streptomycin resistance gene and a classic Tn*10* transposon containing the genes that confer tetracycline resistance. However, we noted that the *papA* gene of every *papA*-negative APEC strain was not interrupted by an 11,104-bp contig. It is likely that transposons bearing antibiotic resistance genes have inserted within *pap* gene cluster of some APEC strains, and such genetic events may have been selected for by antibiotic use.

Keywords
adherence, avian pathogenic Escherichia coli, P fimbriae, papA, pap operon, transposon, virulence

Disciplines
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papA Gene of Avian Pathogenic *Escherichia coli*

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SUMMARY. P fimbrial adhesins may be associated with the virulence of avian pathogenic *Escherichia coli* (APEC). However, most APECs are unable to express P fimbriae even when they are grown under conditions that favor P fimbrial expression. This failure can be explained by the complete absence of the *pap* operon or the presence of an incomplete *pap* operon in Pap-negative APEC strains. In the present study, we analyzed the *pap* operon, specifically the *papA* gene that encodes the major fimbrial shaft, to better understand the *pap* gene cluster at the genetic level. First, by PCR, we examined a collection of 500 APEC strains for the presence of 11 genes comprising the *pap* operon. Except for *papA*, all the other genes of the operon were present in 38% to 41.2% of APEC, whereas the *papA* was present only in 10.4% of the APEC tested. Using multiplex PCR to probe for allelic variants of *papA*, we sought to determine if the low prevalence of *papA* among APEC was related to genetic heterogeneity of the gene itself. It was determined that the *papA* of APEC always belongs to the F11 allelic variant. Finally, we sequenced the '*papA* region' from two *papA*-negative strains, both of which contain all the other genes of the *pap* operon. Interestingly, both strains had an 11,104-bp contig interrupting *papA* at the 281-bp position. This contig harbored a streptomycin resistance gene and a classic Tn*10* transposon containing the genes that confer tetracycline resistance. However, we noted that the *papA* gene of every *papA*-negative APEC strain was not interrupted by an 11,104-bp contig. It is likely that transposons bearing antibiotic resistance genes have inserted within *pap* gene cluster of some APEC strains, and such genetic events may have been selected for by antibiotic use.

RESUMEN. El gene *papA* de la *Escherichia coli* patógena para las aves.

Las adhesinas P fimbriales pueden estar asociadas con la virulencia de la *Escherichia coli* patógena para las aves (APEC). Sin embargo, la mayoría de estas cepas de *E. coli* son incapaces de expresar fimbrias P, incluso cuando se cultivan en condiciones que favorecen la expresión de estas fimbrias P. Esta falta en la expresión se explica por la ausencia completa del operón *pap* o por la presencia de un operón *pap* cepas *pap* negativas de *E. coli* patógena para las aves. En el presente estudio, se analizó el operón *pap*, específicamente el gen *papA* que codifica al eje fimbrial mayor, para entender mejor el grupo de genes *pap* a nivel genético. En primer lugar, mediante PCR, se analizó una colección de 500 cepas de *E. coli* patógena para las aves para determinar la presencia de 11 genes que están comprendidos en el operón *pap*. Con excepción del gene *papA*, todos los otros genes del operón se encontraron presentes en el 38% al 41.2% de las cepas de *E. coli* patógena para las aves, mientras que *papA* estaba presente sólo en el 10.4% de las cepas de *E. coli* patógena para las aves analizadas. Mediante la utilización de un método de PCR múltiple para detectar variantes alélicas de *papA*, se trató de determinar si la baja prevalencia de *papA* entre las cepas de *E. coli* patógena para las aves estaba relacionada con la heterogeneidad genética del mismo gen. Se determinó que el gene *papA* de *E. coli* patógena para las aves siempre pertenece a la variante alélica F11. Finalmente, se secuenció la ''región *papA*'' de dos cepas *papA*-negativas, las cuales contienen todos los otros genes del operón *pap*. Curiosamente, las dos cepas presentaron un contig de 11,104 pares de bases que interrumpían al gene *papA* en la posición 281-pb. Este contig albergaba un gen de resistencia a la estreptomicina y un transposón clásico Tn*10* que contiene los genes que confieren resistencia a la tetraciclina. Sin embargo, hemos observado que el gen *papA* de todas las cepas de *E. coli* patógena para las aves *papA* negativas no estaba interrumpido por un contig 11,104 pares de bases. Es probable que los transposones que contienen genes de resistencia a los antibióticos se ha insertado dentro del conjunto de genes de *pap* de algunas cepas de *E. coli* patógena para las aves, y tales eventos genéticos pueden haber sido seleccionados por el uso de antibióticos.

Key words: adherence, avian pathogenic *Escherichia coli*, P fimbriae, *papA*, *pap* operon, transposon, virulence

Abbreviations: APEC = avian pathogenic *Escherichia coli*; ddH_2O = double distilled water; ExPEC = extraintestinal pathogenic *E. coli*; PAI = pathogenicity island; *pap* = pyelonephritis-associated pilus

The Pap (pyelonephritis-associated pilus) or P fimbria is considered to be an important virulence marker of extraintestinal pathogenic *Escherichia coli* (ExPEC), a group of *E. coli* that includes avian pathogenic *E. coli* (APEC) and are responsible for a diverse spectrum of extraintestinal diseases in poultry (42,46). These fimbriae are encoded by the *pap* gene cluster which consists of 11 genes arranged into an operon (15). The most upstream (5′) genes of the operon, the *papI* and *papB* genes, encode proteins responsible for environmental regulation and phase variation in expression of P fimbriae (1,37). The *papA*, *papH*, *papE*, *papF*, *papG*, and *papK* genes encode the six different subunits of the fimbriae (1,28,29,30) with *papA* encoding the major fimbrial shaft. The corresponding gene product of *papD* is a molecular chaperone that binds with the subunit proteins during their translocation in order to protect them from improper polymerization and proteolytic degradation (8,16,23). *papC* encodes an usher that receives the subunit–chaperone complexes and assembles them into the architecturally distinct fibers (8,23). Likewise, the gene product of *papJ* also functions as a molecular chaperone and is believed to be required for the integrity and correct assembly of *papA* subunits in the pilus (49).

P fimbriae of ExPEC are antigenically diverse, which has been attributed to the peptide sequence variability of *papA*. PapA is present in hundreds to thousands of identical copies in each fimbria, making it the major component of the fimbrial shaft (12,38). According to the classification system of Ørskov, there are 11 different serological variants of PapA, namely F7-1 to F7-2 and F8

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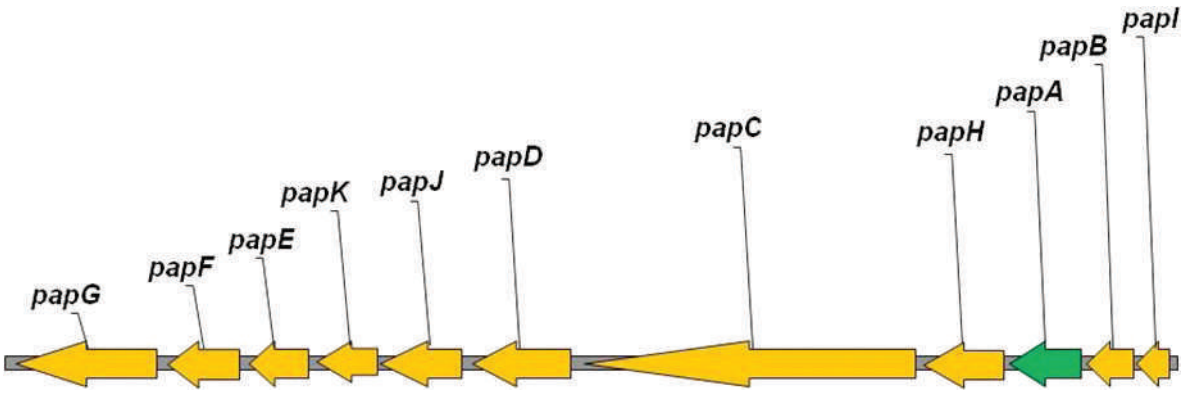

Fig. 1. Schematic diagram describing the genetic organization of pap operon of *papA*$^+$ strain APEC O1 (adapted from Kariyawasam et al. [25]). The least-abundant gene of *pap* gene cluster, the *papA*, is color-coded in green.

to F16 (38). Recently, Johnson *et al.* (20) identified two more PapA variants using a multiplex PCR (17,20,21). Although some UPEC strains such as CFT073 and UPEC536 carry more than one *pap* operon, analysis of the only published and completed sequence of an APEC genome revealed that it possessed only a single *pap* operon contained within a pathogenicity island (PAI) (14,22, 25,44,50). The *pap* operon of the sequenced APEC strain (GenBank accessions DQ095216 and CP000468) showed 96%–99% homology to the *pap* operons of human UPEC (GenBank accessions CP000243, AE014075, and CP000247) at the nucleotide level. Pap fimbriae mediate mannose-resistant Gal(α1-4)Gal-specific binding to glycolipid isoreceptors on host epithelial cells (17,18,19,21,28). This binding is conferred by the pilus tip adhesin encoded by *papG* (28,34). Three different allelic variants of PapG, namely I, II, and III, exhibiting different host cell receptor specificities, have been identified in ExPEC (17,18,19,21,48). However, as is the case with PapA, only a single variant of PapG, PapGII, occurs among APEC (17,18,19,21,42,43,48).

Pourbakhsh *et al.* (39) demonstrated that P fimbriae are expressed in the lower respiratory tract and internal organs of infected chickens, whereas F1 fimbriae are expressed in the upper respiratory tract (41). This suggests that P fimbriae may be required for APEC binding to the lower respiratory tract and subsequent systemic spread, which often leads to polyserositis, but may not play a significant role in APEC binding to the upper respiratory tract or in APEC's initial colonization. Such observations suggest that APEC's P fimbrial expression is controlled through a phase variation mechanism (11,28,35,39,40,41). Most of the genes constituting the *pap* gene cluster are present in 30%–42% of APEC, but the *papA* that encodes the major fimbrial shaft is present in only 6%–7.5% of APEC (9,24,42,43). The significance of the incomplete *pap* operon of APEC is unknown and no study has investigated the likelihood that the PapG tip adhesin mediates binding in the absence of a complete fimbrial structure. Previous reports demonstrated the possibility of using a PapG subunit vaccine to control avian colibacillosis (27). However, the very low prevalence of *papA* among APEC brings into question the spectrum of coverage afforded by a vaccine that targets P fimbriae. As revealed by molecular, morphologic, biochemical, serologic, and functional characterizations of PapA, APEC possess only the F11 allelic variant (11,42,43,51).

The present study was carried out to ascertain the molecular basis for the low prevalence of *papA* as compared to other genes of the *pap* operon in APEC. First, we examined a collection of APEC for the presence of 11 genes of the *pap* operon by PCR. Then, we used a multiplex PCR assay that detects all the known PapA allelic variants of ExPEC including the two variants that were discovered recently. Finally, we sequenced the region between the *papB* and *papH* genes (the region that contains a cryptic *papA* in *papA*-negative strains of APEC) from two well-characterized APEC isolates possessing all the genes of *pap* operon except for *papA*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All the APEC isolates used in the study have been isolated from chickens and turkeys with clinical signs of colisepticemia. These APEC strains originated from different farms and flocks throughout the United States and have been described elsewhere (42,43). The other bacterial strains used in the study are described throughout the manuscript. Bacteria were maintained as glycerol stocks at −80 C and routinely grown in Luria-Bertani agar or broth.

PCR screening of APEC for the genes of the *pap* operon. A collection comprising 500 isolates of APEC was used in the gene prevalence study. These isolates were screened for all 11 genes constituting the *pap* operon (Fig. 1). Crude DNA extracts of bacteria were prepared by the rapid boiling method for use as DNA templates for PCR. Briefly, 1-ml volumes of overnight cultures of bacteria were centrifuged; cell pellets were suspended in 200 µl of double-distilled water (ddH_2O) and heated at 100 C for 10 min. The DNA extracts were maintained at −20 C until use. Extracted DNA was amplified in 25-µl reactions containing 0.5 µM each of forward and reverse primers (Integrated DNA Technologies, Commercial Park, Coralville, IA), listed in Table 1, 0.2 mM each deoxynucleotide (USB, Cleveland, OH), 0.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 2.5 µl of 10× PCR buffer (Invitrogen), 1.5 mM magnesium chloride ($MgCl_2$; Invitrogen), 2 µl of DNA template, and enough ddH_2O to bring the final volume to 25 µl. The PCR reactions of 30 cycles were carried out in a Mastercycler EP machine (Eppendorf, Westbury, NY). The reaction conditions were initial incubation at 94 C for 3 min followed by 30 cycles of 94 C for 45 sec, 59 C for 30 sec, and 72 C for 45 sec and a final extension at 72 C for 5 min. The PCR products were separated in a 1.5% agarose gel, stained with ethidium bromide, and photographed using a gel documentation system. DNA extracted from APEC O1 (O1:K1), which is known to possess the complete *pap* operon containing PAI, the PAI $I_{APEC\text{-}O1}$, and ddH_2O were used as the positive and negative controls, respectively.

Multiplex PCR to detect allelic variants of *papA*. The same crude DNA extracts of bacteria used for the gene prevalence studies were used for the template to detect allelic variants of *papA*. Multiplex PCR was carried out in 3 separate reactions (multiplex PCR 1, 2, and 3) according to the method developed by Johnson *et al.* (20) using the oligonucleotide primers (Integrated DNA Technologies) listed in Table 2. The 25 µl of PCR reaction mixture contained 2.5 µl of 10× PCR buffer (Invitrogen), 0.8 mM of each deoxynucleotide, 2.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA), 0.8 mM $MgCl_2$ (Invitrogen), 2 µl of crude DNA extract, the primers required for each

Table 1. Primers used to study the prevalence of genes of the *pap* operon in APEC.

Primer name	Targeted gene	Amplicon size (bp)	Primer sequence (5′-3′)
PapAF	*papA*	421	GCTGCTCCAACTATTCCACAG
PapAR			ACTGCAGAAAAAGCACCTTCA
PapBF	*papB*	245	AATGCCGACGACTCATCTGT
PapBR			GTACTGTTGCCCGGCTCTAT
PapCF	*papC*	1614	TGTTTACTGGCATGATGGTCA
PapCR			ACGCCGAAAGACGTATATCCT
PapDF	*papD*	517	TCCTCTGCCTGCTTTTCACT
PapDR			TTGTTATATCCGGGGCAGAC
PapEF	*papE*	435	TATCCAAGTTTGGCATGAAGTG
PapER			AGGTTTGTGTCTTCCGGTAATG
PapFF	*papF*	364	GGGCACTGAAGTAAAGGTGAAC
PapFR			GCAGATTAACATCAGGGGAAAT
PapGF	*papG*	801	CCCAGCTTTGTTATTTTCCTTG
PapGR			TTCTTACCATGGCTGTATGTCG
PapHF	*papH*	374	GGTATTGCAGGCATTACTTTCC
PapHR			GAATACTGGGGAGAAGAACACG
PapIF	*papI*	121	TTTCTGAACAGGCATGATGG
PapIR			GTGAGCGCTGAACCATACCT
PapJF	*papJ*	535	TTTATGCTCAGACGCAGCATGG
PapJR			TTGCACTGTCGCTGAGTGGTTT
PapKF	*papK*	397	TAACCTTTTCCCCCTGGTCT
PapKR			GGCGCTCTTTTACTGTTTGC

multiplex PCR (Integrated DNA Technologies), and ddH_2O to bring the final volume to 25 μl. All 3 reactions used universal primer as the forward primer. Multiplex PCR 1 used primers 15r, 13r9, 14r, 11r, and F536 as the reverse primers. Multiplex PCR 2 used primers 9r, 7-1r, F48, 8r, and 12r as the reverse primers, whereas multiplex PCR 3 used 12er9, 10r4, 16r1, and 7-2r as the reverse primers. Each reaction used the primers 13r9, 14r, 10r4, 16r1, and 7-2r at a concentration of 0.6-μM concentrations and the primers 15r, 13r9, 11r, F536, 9r, 7-1r, F48, 8r, 12r, and 12er9 were used at a concentration of 0.3 μM. Amplification was performed in a Mastercycler EP machine (Eppendorf) according to the following parameters: 12 min at 95 C, 24 cycles of 30 sec at 94 C, 30 sec 67 C, 90 sec at 72 C, 10 min at 72 C, and a final hold at 4 C. The products were then separated in a 2% agarose gel and a 100-bp ladder (Invitrogen) was used as a size reference. The *E. coli* reference strains, CFT073 (positive for F7-1 and F7-2 allelic variants) originated from Dr. Harry Mobley, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, V30b (positive for F8 allelic variant), H9 (positive for F9, F12, and F48 allelic variants), H1 (positive for F10, F11, and F16 allelic variants), CP9 (positive for F13 and F14 allelic variants), and DD001 (positive for F536 allelic variant) were all kindly provided by Dr. J. R. Johnson (Medical Service, VA Medical Center and Department of Medicine, University of Minnesota, Minneapolis, MN, USA) for use as positive controls.

Analysis of the region between *papB* and *papH* in two *papA*-negative strains. Genomic DNA from APEC15 and APEC20 was extracted with the Genomic DNA Extraction Kit (Qiagen, Valencia, CA). These two strains carry each of the genes of the *pap* operon, except for *papA*, and were virulent in 2-wk-old chickens as assessed by an intratracheal infection model. Detailed characteristics of both isolates are listed in Table 3. Two complementary PCR approaches (long-range amplification and genome walking) were used to sequence the region between *papB* and *papH* (*papB* → *papH* region) which contains a cryptic *papA* gene in *papA*-negative strains. First, a PCR that utilized primers PapBF and PapHR (Table 1), and the LongRange PCR Kit (Qiagen) was used to amplify the *papB* → *papH* region from APEC15 and APEC20. The PCR reaction was carried out according to the manufacturer's recommendations. Briefly, a 50-μl volume of amplification reaction consisting of 5 μl of 10× LongRange PCR buffer with Mg^{2+} (2.5 mM final concentration of $MgCl_2$, 500 μM of each deoxynucleotide, 0.4 μM each of primers PapBF and PapHR; Table 2), 2 U of LongRange PCR enzyme mix, 10 μl of template DNA, and ddH_2O to bring the volume to 50 μl. PCR conditions were as follows:

Table 2. Primers used to detect allelic variants of *papA*.

Primer name[A]	Targeted allele	Size of the amplicon (bp)	Primer sequence (5′-3′)
Universal forward	All *papA* variants		GGCAGTGGTGTCTTTTGGTG
15r[1]	F15	457	GCTACATTCTTGCCACTTGC
13r9[1]	F13	399	GGGTATTAGCATCACCTTCGGAG
14r[1]	F14	332	GCAGCATATCTTTATTGTTCCC
11r[1]	F11	278	GGCCCAGTAAAAGATAATTGAACC
F536[1]	F536	257	CCTGGAGCGGCACCAGTGGCT
9r[2]	F9	418	AAGGCCCCGTTGACGTTTT
7-1r[2]	F7-1	377	TTTCACCCGTTTTCCACTCG
8r[2]	F8	253	GTACCACCTACAGCACTTGG
F48[2]	F48	303	GTTCATTGCTCACTAGGATGACCACTA
12r[2]	F12	179	AATTCTTGGGCGTTGAGGATCCA
12er9[3]	F12	393	CCCATCGACAAGACTTGACA
10r4[3]	F10	314	CTCCTCATTATGACCAGAAACCCT
16r1[3]	F16	241	GTTCCCGCTTTATTACCAGC
7-2r[3]	F7-2	185	TTTGGGTTGACTTTCCCCATC

[A]Primers = [1]Constitute the primer pool 1; [2]constitute the primer pool 2; [3]constitute the primer pool 3.

Table 3. Characteristics of APEC15 and APEC20 strains.

Strain	APEC15	APEC20
Serogroup	Rough	O61
Origin	Turkey pericardium	Chicken yolk sac
Genotype[A]	*hlyD*−, *cnf1*−, *cdtB*−, *fyuA*+, *iroN*+, *ireA*+, *iutA*+, *sitA*+, *feoB*+, *cvaC*+, *iucC*+, *iss*−, *traT*+, *papA*−, *papB*+, *papC*+, *papD*+, *papE*+, *papF*+, *papG*+, *papH*+, *papI*+, *papJ*+, *papK*+, *afa*−, *iha*−, *sfa*−, *focG*−, f*imH*+, *ompT*+, *ibeA*+, *tsh*+, *stg*+	*hlyD*−, *cnf1*−, *cdtB*−, *fyuA*+, *iroN*+, *ireA*+, *iutA*+, *cvaC*+, *sitA*+, *feoB*+, *iucC*+, *iss*+, *traT*+, *papA*−, *papB*+, *papC*+, *papE*+, *papD*+, *papE*+, papF+, *papF*+, *papG*+, *papH*+, *papI*+, *papJ*+, *papK*+, *afa*−, *iha*−, *sfa*−, *focG*−, f*imH*+, *ompT*−, *ibeA*+, *tsh*+, *stg*+
Phylogenetic group	A	A

[A]Description of genes (*hlyD* = hemolysin operon gene; *cnfI* and *cdtB* = toxin genes; *fyuA*, *iroN*, *ireA*, *iutA, iucC*, *sitA*, and *feoB* = iron-regulated protein genes; *cvaC* = colicin operon gene; *iss* = increased serum survival gene; *traT* and *ompT* = outer membrane protein genes; *papA* to *papK* = P fimbrial operon genes; *afa* and *iha* = adhesin genes; *sfa* = S fimbrial gene; *focG* = F1C fimbrial gene; *fimH* = Type 1 fimbrial adhesin; *ibeA* = brain microvascular endothelial cell invasion gene; *tsh* = temperature-sensitive hemagglutinin gene; and *stg* = Stg fimbrial gene).

initial denaturation at 93 C for 3 min; 10 cycles of 93 C for 15 sec, 62 C for 30 sec, 68 C for 13 min; 28 cycles of 93 C for 15 sec, 62 C for 30 sec, and 68 C for 13 min with 20-sec increments after the 10th cycle. The product was sequenced using a high throughput DNA sequencing strategy using the Applied Biosystems 3730xl DNA Analyzer at the DNA Facility of Iowa State University. Sequencing was started with the primers PapB1 and PapH2. The subsequent primers for sequencing were designed based on the preceding sequence information. Gaps were filled using a commercial GenomeWalker Universal kit (Clontech, Palo Alto, CA) following the manufacturer's recommendations. This method involves ligation of adaptors to purified uncloned libraries of genomic DNA digested with different restriction enzymes. Briefly, genomic DNA from APEC15 and APEC20 was digested with four different restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, *Stu*I) and the resulting fragments were ligated blunt-end to two adaptor oligonucleotides provided with the kit. The region downstream of *papB* and upstream of *papH* was then amplified with the adapter primers (AP1 and AP2) provided in the kit and two sets of primers specific to the *papB* and *papH* regions in two PCR reactions. Primers for the primary PCR were PapBGW1 (ATCTGGTATTTCTCGCAGACCTCCTTAC) and AP1 or PapHDW1 (TTAACAGTCAGGGTGGGAACCTTTTCTC) and AP1. The conditions for PCR in the Mastercycler EP machine (Eppendorf) were as follows: seven cycles of 25 sec at 94 C and 3 min at 72 C, and 32 cycles of 25 sec at 94 C, 3 min at 67 C, and 7 min at 72 C. The nested PCR used primers PapBGW2 (ACCTACCAGATAGTCCTTCATAGCCAGA) and AP2 or PapHDW2 (CAATACCATTGACGGGTAATGAAGAAG) and AP2. The amplification conditions used were five cycles of 25 sec at 94 C and 3 min at 72 C, and 20 cycles of 25 sec at 94 C, 3 min at 67 C, and 7 min at 67 C. The PCR products were cloned into a pGEMT-Easy vector (Promega) and sequenced at the Iowa State University's DNA facility. The newly defined sequence portions were used to design new primers for use in subsequent amplification steps, and the walking procedure was repeated until the entire sequence was determined.

Screening of APEC possessing all the genes of *pap* operon, except *papA*, for the presence of an 11,104-bp insertion. Fifteen isolates of *papA*-negative APEC that possess other genes of the *pap* operon were tested by PCR to determine whether all these isolates carry an 11,104-bp insertion within the *papA* gene. These isolates were also known to possess *strA*, *strB*, and *tetB* genes. The PCR used primers PapBF and PapHR (Table 1) and the Qiagen LongRange PCR Kit as described under the preceding section.

RESULTS

PCR screening of APEC for the genes of the *pap* operon. Among 500 APEC isolates tested, the prevalence of various *pap* operon genes ranged from 10.4% ($n = 52$) to 41.2% ($n = 206$); see Table 4. The *papA* was detected only in 10.4% ($n = 52$) of the isolates tested whereas the other genes constituting the *pap* operon were detected in 38% ($n = 190$) to 41.2% ($n = 206$) of the APECs tested. In detail, 206 (41.2%) isolates possessed at least one of the genes of the operon. Of the APEC strains tested, 190 (38%) were positive for *papB*, 206 (41.2%) were positive for *papC*, 200 (40%) were positive for *papD*, 202 (40.4%) each were positive for *papE* and *papF*, 196 (39.2%) were positive for *papG*, 194 (38.8%) were positive for *papH*, 190 (38%) were positive for *papI*, 192 (38.4%) were positive for *papJ*, and 195 (39%) were positive for *papK*. The *papC* gene was the most prevalent gene among all 11 genes constituting the *pap* operon, and four of the APEC strains tested in the present study carried only this *pap* gene. When an isolate was positive for any *pap* genes other than *papC* by PCR, that isolate was always positive for at least *papC*.

Multiplex PCR to detect allelic variants of *papA*. Of the 500 APECs tested in the study, 52 (10.4%) strains were found by multiplex PCR to contain a *papA* allele. All *papA*-positive isolates yielded an amplicon of 278 bp in size only with the primer pool 1 that amplified F15 (457 bp), F13 (399 bp), F14 (332 bp), F11 (278 bp), and F536 (257 bp) allelic variants, suggesting that all *papA*-positive APEC strains possessed the F11 PapA allele.

Analysis of the region between *papB* and *papH* in *papA*-negative strains. The long-range PCR amplified a DNA fragment of approximately 11 kb in size (Fig. 2). Continuous sequencing of this fragment was not possible due to the presence of repetitive sequences. Therefore, we used long-range PCR in combination with genome walking to sequence the entire *papB* → *papH* region. The nucleotide sequences of *papB* → *papH* region of APEC15 and APEC20 strains have been deposited in the GenBank database under the accession numbers JF916463 and JF916464, respectively. Sequencing revealed that an 11,104-bp contig is inserted within *papA* at the 281-bp position, totally disrupting its function. This contig contained a classic Tn*10* transposon that carries the

Table 4. Prevalence of *pap* operon genes in APEC.

Gene	Gene product	Total ($n = 250$)	Percentage
papA	Major fimbrial shaft	52	10.4
papB	Regulatory protein	190	38.0
papC	Usher protein	103	41.2
papD	Molecular chaperone	200	40.0
papE	Subunit protein	202	40.4
papF	Subunit protein	202	40.4
papG	Tip adhesin	196	39.2
papH	Subunit protein	194	38.8
papI	Regulatory protein	190	38.0
papJ	Molecular chaperone	192	38.4
papK	Subunit protein	195	39.0

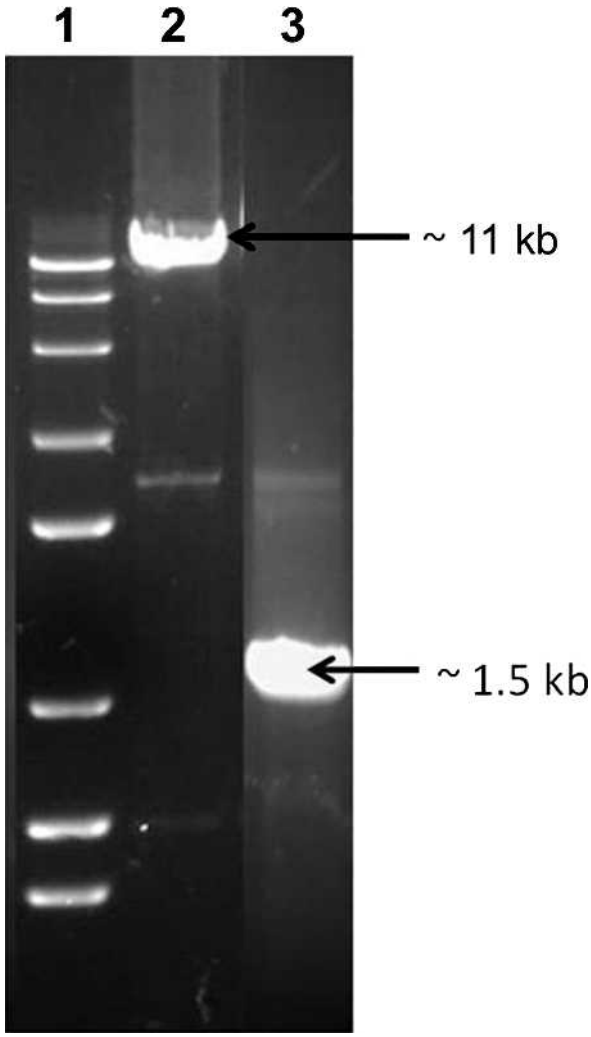

Fig. 2. PCR products obtained from long-range PCR amplification. Lane 1: molecular weight marker; Lane 2: *papB*→ *papH* region of APEC O1 (*papA*$^+$).

tetracycline resistance gene, *tetB*, among other genes (Fig. 3). Tn*10*, which is a composite tetracycline-resistance transposon, consisted of two indirectly repeated copies of the IS*10* insertion sequence (IS) elements flanking the transposon. Within this composite transposon, there were four hypothetical proteins of unknown function. Another intriguing feature of this contig was the presence of a truncated IS*10* sequence followed by the streptomycin resistance genes *strA* and *strB* between the 3′ end of the interrupted *papA* gene and Tn*10*.

Screening of APEC possessing all the genes of *pap* operon, except for *papA*, for the presence of an 11,104-bp insertion. Of the 15 isolates, only 11 isolates contained the expected ~11,000-bp region. Three isolates yielded a PCR product that was less than 11,000 bp, and the remaining two isolates produced a PCR product that was bigger than the expected amplicon size.

DISCUSSION

APEC has become a prominent pathogen of production birds. Existing literature suggests that P fimbriae are important virulence markers of APEC (5,9,43,51). However, *papA* was present only in 10.4% of APECs tested in this study while the other genes of the operon (*papB*, *C*, *E*, *F*, *G*, *H*, *I*, and *J*) were detected at a higher frequency (from 38% to 41.2%). Existing literature also explained this variation in distribution of *pap* genes by suggesting that most APEC lack an intact copy of the P fimbrial operon (9,10,24,42,43). Nevertheless, previous studies have also demonstrated that despite the presence of certain *pap* genes, some APEC do not express P fimbriae even after growth under conditions designed to enhance P fimbrial expression (11). Supposedly, P fimbriae may not essentially be required for APEC pathogenesis but might play a significant role in Pap-positive strains that do not possess other fimbrial adhesins such as Stg (31), AC/I (avian *E. coli* I) (52), and F17 fimbriae (47). Nevertheless, there was no correlation between the serotype of APEC and the presence or absence of the *papA* gene within the *pap* operon (5,9,43,51).

In contrast to the study by Dozois *et al.* (10), which reported that the *papI* gene of APEC is less prevalent than the *papA*, *papC*, or *papG* genes, our results indicated that the *papA* gene is the least prevalent among the genes making up the APEC *pap* operon. A notable difference between these two studies is that Dozois *et al.* (10) used only 13 APEC strains known for their possession of the *papC* gene, whereas the present study used 500 APEC isolates originated from chickens and turkeys, which may have allowed a more representative analysis. Nevertheless, primers used in the present study were designed according to APEC *pap* operon sequences (DQ095216), whereas Dozois *et al.* (10) used primers that were designed based on UPEC *pap* sequences. It is well documented that *papA* of ExPEC from humans, dogs, and cats is antigenically diverse and belongs to one of the 13 allelic variants (13,15,20,38). This allelic variation is determined at the level of the peptide sequence of PapA (38) and is detectable by the nucleotide sequence analysis of the encoding gene (38). Cognizant of this allelic diversity, we assumed that the scarcity of *papA* in APEC, as compared to the other genes of the operon, might be due to the inability of certain *papA*-specific PCR methodologies to detect all allelic variants of *papA*. To test this possibility, we used a multiplex PCR that was designed by Johnson *et al.* (20) to detect all the allelic variants of *papA* including two novel variants. Our results demonstrated that APEC strains possess only one *papA* allele and confirmed earlier findings by other investigators that suggested *papA* of APEC is antigenically closely related to F11 serotype (9,11,51). The current study also proved that *papA* is absent in APECs that harbor an incomplete copy of the *pap* gene cluster.

Next, we were interested in analyzing the *papB* → *papH* region, which corresponds to the *papA* gene in *papA*$^+$ isolates, using two APEC isolates that possess all the genes of *pap* operon except for *papA*. The long-range PCR amplified a DNA fragment of approximately 12 kb in size. Sequencing of the amplified revealed that an 11,104-bp contig, containing a composite Tn*10* transposon (4), is inserted within *papA* at the 281-bp position, totally disrupting its function. This interrupted region contained a tetracycline resistance gene, four hypothetical proteins of unknown function, a truncated IS*10* sequence, and streptomycin resistance genes. However, our study indicated that all *papA*-negative APEC that possess other genes of *pap* operon do not carry this 11,104-bp contig within the *papA* gene. This suggests that more than one mechanism is involved with *papA* gene interruption.

Transposons are mobile genetic elements that play a profound role in microbial evolution by promoting gene inactivation, genome plasticity, new gene introduction, or all of these (2,32,33). According to available literature, they are mostly associated with shuttling adaptive traits such as antimicrobial resistance, virulence, and new metabolic capabilities (2). Further, they are capable of mediating transfer of genetic information between replicons in the same genome, or between different genomes, by means of horizontal gene transfer. Transposition

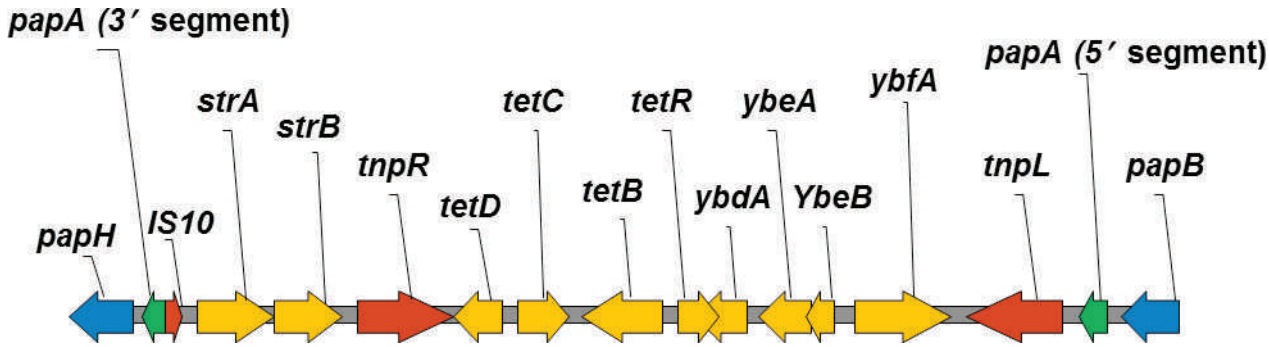

Fig. 3. Schematic diagram showing the genetic organization of the *pap* operon of APEC15 and APEC20 strains. Note the 11,104-bp contig containing streptomycin and tetracycline resistance genes interrupting *papA*. The *papA* gene, which is interrupted due to transposition, is color-coded in green, the two *pap* operon genes flanking the *papA* gene are color-coded in blue, and mobile genetic elements are color-coded in red.

can also convert pathogens to nonpathogenic forms by disrupting genes that are required for virulence (45). Although the exact role of the transposable element present within *papA* is unclear, it is likely to increase genome plasticity and diversity. As is the case with other *E. coli*, diversity and genome plasticity among ExPEC is well documented (3,6,7,26,36) and is thought to provide an evolutionary advantage associated with acquisition of foreign DNA or deletion of existing genetic information. Because the genetic regions vulnerable to such changes are frequently subjected to excisions, rearrangements, and transfers, they contribute to the rapid evolution of *E. coli* variants, resulting in the development of novel strains and even pathotypes. Perhaps, Tn*10* may have been selected for in APEC because the use of tetracyclines as growth promoters and therapeutics became common practice in poultry.

In a separate study, we showed that prevalence of the *papA* gene in APEC decreased from the 1980s to the 1990s (from 16.84% to 6.32%), while no statistical difference in the occurrence of the other *pap* genes was detected between APEC isolated over these two decades (24). Although the exact role of transposable elements in the *pap* operon of APEC is unclear, it is likely that incorporation of antimicrobial resistance genes into the genome would have provided a survival advantage to bacteria. Both APEC15 and APEC20 possess other adhesins, such as Stg fimbriae, which are known to play a significant role in APEC's colonization of the respiratory tract and pathogenesis. It can be speculated that the role of the *pap* operon in adherence may be served by other bacterial traits and, therefore, the possession of antimicrobial resistance genes is more beneficial to a pathogen's survival, especially in the face of antimicrobial pressure.

In summary, the *pap* operon genes are either incomplete or completely absent in a majority of APEC—indicating that they do not contain a functional copy of the P fimbrial operon. The least-abundant gene of the *pap* operon among APEC is *papA*. If present in APEC, *papA* is always of the F11 type. In some APEC, *papA* is interrupted by transposition, thus preventing synthesis and expression of the PapA subunit and typical P fimbriae. In the two strains of APEC (APEC15 and APEC20) examined in this study, the *papA* region contains an insert of 11,104 bp in size that harbors tetracycline and streptomycin resistance genes.

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